REMARKS

The Office Action of March 27, 2002 presents the examination of claims 1-10, 14, and 15. No amendments to the application are made and thusly no new matter is inserted into the application.

Request for Interview

If, for any reason, the present invention is not placed into condition for allowance upon entry of this Reply, the Examiner is respectfully requested to contact Kristi L. Rupert, Ph.D. (Reg. No. 45,702) at 703-205-8000 to schedule a personal interview at the Examiner's convenience.

Rejection under 35 U.S.C. § 112, first paragraph

The Examiner maintains the rejection of claim 3 under 35 U.S.C. § 112, first paragraph for allegedly not being described in the specification. Applicants respectfully traverse. Reconsideration of the claims and withdrawal of the instant rejection are respectfully requested.

Claim 3 is directed to the method of claim 2, further comprising determining the presence, position, and type of mutation and categorizing biological aggressiveness and/or metastatic potential of the neoplasia based upon the presence,

position, and type of mutation, wherein said neoplasia is breast cancer, and wherein a mutation in a conserved region II and V of p53 is indicative of poor patient outcome whereas a mutation in a conserved region III and IV is indicative of positive patient outcome.

The Examiner apparently disagrees that one skilled in the art would know that a frameshift or nonsense mutation would be more detrimental than a missense mutation. Specifically, the Examiner writes, "Without guidance or exemplification, one of ordinary skill in the art would not know which type of mutation in conserved region II and V would give rise to a [*sic*] affect binding or transactivation mutation."

In response to the Examiner's remarks, Applicant submit herewith a journal article, Sjögren et al. "The p53 Gene in Breast Cancer: Prognostic Value of Complementary DNA Sequencing Versus Immunohistochemistry," *Journal of the National Cancer Institute*, Vol. 88, No. 3/4, 1996 illustrating how mutations which affect DNA binding or transactivation are typically frameshift or nonsense mutations.

In figure 1 of said article, there is a graphic illustration of where mutations are found and the mutation

types. There are essentially four types of mutations that may occur:

1. Missense mutations, where a codon is mutated causing a change in the corresponding amino acid.

2. Nonsense mutations, where a codon is mutated into a stop codon which terminates protein expression at the mutated codon.

3. Deletions which are in-frame, where nucleotides are deleted in multiples of 3 which causes corresponding loss of amino acid(s), or out-of-frame, where nucleotides are deleted in numbers where a premature stop codon is created somewhere downstream of the mutation. In such cases, the expressed protein almost never corresponds to the normal protein.

4. Insertions which are in-frame, where nucleotides are inserted in multiples of 3 which causes corresponding addition of amino acid(s), or out-of-frame, where nucleotides are added in numbers where a premature stop codon is created somewhere downstream of the mutation. Again, in such cases, the expressed protein almost never corresponds to the normal protein.

Referring to figure 1 of the paper, it is quite clear that missense mutations are concentrated in the very central, DNA-

binding region of the p53 protein. Other types of mutations are much more widely distributed.

On page 3, lines 9-19 of the specification, it is stated that approximately 70% of mutations in p53 are missense mutations that change the identity of an amino acid and alter the confirmation and stability of p53. Further, on page 7, lines 32-38, it is stated that mutations in p53 that give rise to transcriptional stop signals and a truncated protein prevents p53 from employing its DNA proof-reading role. Finally, on page 8, lines 3-10, it is stated that mutations detrimental to the patient are those which affect the DNA binding or transactivation, whereas those mutations less harmful for the patient are amino acid changes not greatly affecting structure or function of p53.

Therefore, contrary to the Examiner's remarks, one skilled in the art, given the information disclosed in the specification, would be advised that a frameshift or nonsense mutation would be more detrimental to a cancer patient, whereas a missense mutation would be less detrimental to the cancer patient.

Thus, contrary to the Examiner's assertions, the prognosis of neoplasia based on the "type" of mutation is indeed described

in the specification so as to reasonably convey to one skilled in the art that the present Inventors had possession of the claimed subject matter at the time of filing.

As these remarks address and overcome the issues of written description raised by the Examiner, Applicants respectfully request withdrawal of the instant rejection.

Rejection under 35 U.S.C. §§ 102, 103

The Examiner maintains the rejection of claim 15 under 35 U.S.C. § 102(e) for allegedly being anticipated by Vogelstein '676 (USP 5,527,676). The Examiner also maintains the rejection of claims 1, 2, 4-10, and 14 under 35 U.S.C. § 103(a) for allegedly being obvious over Vogelstein '676, in view of Elledge et al. and Callahan et al., and further in view of Hedrum et al. Applicants respectfully traverse. Reconsideration of the claims and withdrawal of the instant rejection are respectfully requested.

Claim 15 recites a method for prognostication of the development of neoplasia in a human patient having a neoplasia comprising: a) determining the nucleotide sequence of exons 2-11 of a cancer-related p53 nucleic acid derived from a human neoplastic tissue or body fluid; b) analyzing the entire

nucleotide sequence determined in step a) for the presence of mutations; and c) classifying the neoplasia into different subgroups depending on the presence or absence of a mutation; and d) prognosticating the development of the neoplasia by analyzing the results of step c) only, wherein said results are indicative of patient survival. The Examiner asserts that Vogelstein '676 anticipates claim 15 because "Vogelstein teaches sequencing all of the p53 gene" and "it is inherent that 'part' of the gene that is sequenced encompasses exons 2-11."

Vogelstein '676 fails to disclose a method for prognostication of the development of neoplasia in a human patient. Vogelstein '676 merely discloses a method for diagnosing a neoplastic tissue of a human (see column 1, lines 51-52). The difference between the *prognostication* of the development of neoplasia and the *diagnoses* of neoplastic tissue are quite different to one skilled in the art, such that the disclosure of one does not destroy novelty of the other.

Typically, the diagnosis of malignant disease on solid tumors is made prior to surgery by conventional means (biopsies, fine needle aspirates) and examined by pathologists looking for typical cellular characteristics to establish diagnosis. In breast cancer at least, this is the sole basis for diagnosis and

initial surgical treatment. After surgical intervention has been made, the primary tumor can be further examined, for instance by: detecting allelic loss of certain defined genes, detecting expression of certain proteins by immunohistochemistry, or by DNA sequencing the entire or parts of genes for mutations.

On the other hand, in order to progosticate a disease i.e., to make a prognosis on how the disease will develop in absence of any additional treatment, as recited in claim 15 in the present application, a number of factors have to be taken into account. In breast cancer, factors that contribute with prognostic information are for instance, nodal status and tumor size. The present invention utilizes the mutational status of the p53 gene in the tumor cells to prognosticate the development of neoplasia. If the prognosis of disease for a given patient is poor, more aggressive treatment is prescribed.

Vogelstein '676 fails to disclose or suggest the prognostication of the development of neoplasia. Further, Vogelstein '676 fails to associate the metastatic potential of the neoplasia based upon the presence, position, and type of mutation. Nor does Vogelstein '676 suggest that certain mutations in p53 are indicative of poor patient outcome.

Instead, Vogelstein '676 merely provides the skilled artisan with methods for assessing p53 in human tumors.

In summary, Vogelstein '676 fails to disclose a method for prognostication of the development of neoplasia in a human patient. As such, Vogelstein '676 fails to anticipate or render obvious the present invention. Further, Elledge et al. and Callahan et al. fail to detect p53 mutations by sequencing exons 2-11 of the gene, whereas Hedrum et al. merely teaches the sequencing of exons 4-9. Thus, absolutely no reference teaches a method for prognostication of the development of neoplasia by sequencing exons 2-11 of p53.

As such, the present invention is not unpatentable over the combination of references cited by the Examiner. Withdrawal of the instant rejection is therefore respectfully requested.

Summary

Overall, the present invention possesses significant patentable features that the cited prior art references do not possess. Furthermore, Applicants submit the instant claims are fully in compliance with 35 U.S.C. § 112, first paragraph. All of the present claims define patentable subject matter such that this application should be placed into condition for allowance.

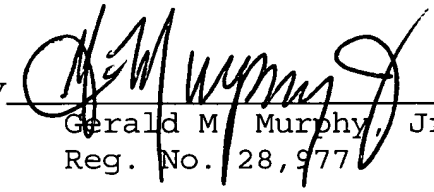
Favorable action on the merits of the present application is thereby requested.

Pursuant to the provisions of 37 C.F.R. §§ 1.17 and 1.136(a), the Applicants hereby petition for an extension of three (3) months to September 27, 2002, in which to file a reply to the Office Action. The required fee of $920.00 is attached to the Notice of Appeal, which is being filed concurrently herewith.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §§1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By 

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Attachment: Sjögren et al. "The p53 Gene in Breast Cancer: Prognostic Value of Complementary DNA Sequencing Versus Immunohistochemistry," *Journal of the National Cancer Institute*, Vol. 88, No. 3/4, 1996

Adjuvant therapy is commonly used in the management of cancer. However, its role in the management of prostate cancer remains to be defined (*14*). Reports of low re-treatment rates after radical prostatectomy may have led many patients to believe that radical prostatectomy alone is sufficient for managing their prostate cancer. Since the likelihood of requiring additional cancer treatment after initial therapy might influence their treatment decisions, it is important that patients be informed of the re-treatment rates based on the experience of men treated in a spectrum of community settings as well as men treated at selected academic medical centers. Given the wide use of follow-up cancer treatments after initial surgery and the uncertainties concerning the value of these treatments (*14*), the effectiveness of various follow-up treatment strategies after radical prostatectomy warrants further investigations.

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Notes

[1] *Editor's note:* SEER is a set of geographically defined, population-based central tumor registries in the United States, operated by local nonprofit organizations under contract to the National Cancer Institute (NCI). Each registry annually submits its cases to the NCI on a computer tape. These computer tapes are then edited by the NCI and made available for analysis.

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The p53 Gene in Breast Cancer: Prognostic Value of Complementary DNA Sequencing Versus Immunohistochemistry

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Background: **Mutations in the p53 tumor suppressor gene (also known as TP53) have been detected in a wide variety of human cancers. In breast cancer, the presence of p53 gene alterations has been associated with worse prognosis. *Purpose:* We compared a complementary DNA (cDNA)-based sequencing method and an immunohistochemical (IHC) method for their abilities to detect p53 mutations in breast cancer specimens. In addition, we determined the prognostic value of information obtained when these two methods were used. *Methods:* Specimens from 316 primary breast tumors were evaluated for the presence of mutant p53 protein by use of the mouse monoclonal antibody Pab 1801 (that recognizes both wild-type and mutant forms of p53) and standard IHC methods. In addition, the entire coding region of p53 genes expressed in these tumors was screened for mutations by combining reverse transcription, the polymerase chain reaction, and DNA sequencing. Probabilities for overall survival (OS), breast cancer-corrected survival (BCCS; death from breast cancer is the considered event), and relapse-free survival (RFS) were estimated by use of the Kaplan–Meier method, and survival curves for different patient subgroups were compared by use of the logrank method. All reported *P* values are from two-sided tests. *Results:* Sixty-nine (22%) of 316 tumors had p53 gene mutations detected by the cDNA-based sequencing method; only 31 (45%) of these mutations were located in evolutionarily conserved portions of the p53 coding region. Sixty-four tumors (20% of the total) had elevated levels of p53 protein as detected by IHC, suggesting the presence of mutations. Of the sequencing-positive tumors (i.e., p53 mutant), 23 exhibited negative IHC reactions, indicating that IHC failed to detect 33% of the mutations. Furthermore, 19 of the IHC-positive tumors were sequencing negative (i.e., p53 wild-type), suggesting a 30% false-positive frequency with IHC. Four tumors (1.3% of the total) could not be analyzed by the cDNA-based sequencing method, and three tumors (1% of the total) could not be analyzed by IHC. The 5-year estimates for RFS, BCCS, and OS were significantly shorter for patients with p53 sequencing-positive tumors than for patients with sequencing-negative tumors (*P* = .001, *P* = .01, and *P* = .0003, respectively). Patients with IHC-positive tumors showed reduced survival in all three categories when compared with those with IHC-negative tumors, but the differences were not statistically significant. *Conclusions:* Use of a cDNA-based sequencing method to determine the status of the p53 gene in primary breast cancers yielded better prognostic information than IHC performed with the Pab 1801 monoclonal antibody. [J Natl Cancer Inst 1996;88:173-82]**

Alteration of the tumor suppressor gene p53 (also known as TP53) is considered to be a critical step in the development of many human cancers (*1,2*). Changes in this gene have been detected in a wide range of human tumors, including breast cancers (*3*). The p53 gene is located on chromosome 17p, and its product is a nuclear phosphoprotein. The p53 protein has been identified as a transcription factor with sequence-specific DNA-binding properties and an ability to regulate entry into S phase of the cell cycle (*1,4,5*). The p53 protein has also been shown to influence the induction of apoptosis in malignant cells (*6*).

In breast cancer, research has focused on patients with primary, node-negative breast disease, and alterations in the p53 gene have been associated with worse prognosis (*7-9*).

Previous studies (*8,10-18*) evaluating p53 status in cancer have used single-stranded conformation polymorphism analysis (SSCP), DNA sequence analysis, or immunohistochemistry (IHC). Detection of p53 mutations by IHC is based on the ac-

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See "Notes" section following "References."

cumulation of mutant p53 protein in cells due to conformational changes in the p53 polypeptide that result in increased stability (*4*,*19*). Wild-type p53 protein has a short half-life and is not usually detected by IHC. However, the accuracy of detecting p53 mutations by IHC has been questioned. A recent study (*20*) comparing five different anti-p53 antibodies revealed positive IHC staining in 18%-35% of the tumors examined if individual antibodies were used and in 45% of the tumors if a cocktail of two of the antibodies (i.e., antibody 240 and Pab 1801) was used. Even if the true mutation status of the p53 gene is not revealed by IHC, it has been suggested that this methodologic approach may provide information more reflective of the functional status of the p53 protein than that provided by molecular biological techniques such as DNA sequencing (*21*) and, therefore, might be more effective in defining patients with poor prognosis.

In this study, we compared the prognostic value of screening for p53 gene mutations in breast cancer specimens using IHC (employing the monoclonal antibody Pab 1801) with that of complementary DNA (cDNA)-based sequence analysis of the complete p53 coding region. The cDNA sequences used in this study were determined as part of an effort to evaluate p53 gene status in the primary tumors of 316 patients with breast cancer in relation to adjuvant therapy and prognosis (*22*).

Materials and Methods

Study Materials and Patient Population

Tumor material from 316 consecutive patients operated on for primary invasive breast cancer in Uppsala County (Sweden) from January 1, 1987, through December 31, 1989, was used in this study. The tumors were collected at the Department of Pathology, University of Uppsala, Akademiska sjukhuset, Sweden. This department contained the only laboratory for histopathology in Uppsala County during the study period. All breast cancer samples were sent directly, unfixed and fresh, from the operation theater to the Department of Pathology.

Patient age at diagnosis ranged from 28 to 94 years, with a median age of 63 years. Tumor sizes ranged from 2 to 130 mm, with a median size of 20 mm. Lymph node metastases were detected in 97 (31%) of the 316 patients.

Of the 316 patients, 111 were diagnosed via a mammography screening program initiated in 1988, which invited all women in Uppsala County aged 40-74 years to participate.

Patient records were reviewed with regard to relapse information and date of death without knowledge of the p53 status of the tumors. Details about primary therapy, including radiotherapy, and relapse therapy were also recorded. All fatal outcomes were studied, and causes of death were divided into three categories: 1) death caused by breast cancer, 2) death with signs of active breast cancer, and 3) death from other causes without signs of relapse. These clinical data were analyzed together with prospectively recorded data on lymph node status and p53 data from IHC and cDNA sequencing.

Therapy and Clinical Follow-up

Locoregional therapy. Primary surgical therapy consisted of either sector resection or modified radical mastectomy; in both instances, this was combined with axillary lymph node dissection. Axillary lymph node status information is missing for 13 patients for whom nodal exploration was not performed because of high age or the presence of other serious concomitant disease.

Patients with lymph node-positive disease or tumors larger than 20 mm in diameter located in the medial or central area of the breast received locoregional radiotherapy, except for 19 women who did not receive such therapy because of high age or the presence of concomitant or metastatic disease. Patients who underwent sector resection were routinely given radiotherapy, except for those who participated in two randomized studies exploring the efficacy of postoperative radiotherapy. [One of these studies has been published (*23*)].

Systemic adjuvant therapy. Systemic adjuvant therapy was offered routinely to all patients with lymph node-positive disease. Premenopausal women received intravenous adjuvant polychemotherapy consisting primarily of six to nine courses of intravenous cyclophosphamide, methotrexate, and 5-fluorouracil. When radiotherapy was given concurrently, only cyclophosphamide was administered. Tamoxifen was given to postmenopausal women with lymph node-positive disease. Tamoxifen was also given to women with stage II tumors who were lymph node negative as part of a randomized trial. Individualized therapeutic strategies were used for patients with primary inoperable disease or metastatic disease.

Follow-up. All patients treated for breast cancer in Uppsala County were seen on a regular outpatient basis at increasing intervals of time each year for at least 5 years; after 5 years, they were seen on a yearly basis until 10 years of follow-up had been completed. A few patients were followed at institutions other than the University of Uppsala; they were referred back to the University of Uppsala (Department of Oncology) on relapse, since this institution has the only clinic for oncology in Uppsala County. The routine follow-up evaluation consisted of a clinical examination. Blood tests and x-ray procedures were performed when indicated. Women aged 40-74 years had mammography checkups through the screening program.

Handling of Tumor Material

Each freshly isolated tumor was divided into two equal portions by use of a disposable scalpel. Slices were taken from the outer, viable, cellular region of one half and frozen in isopentane for later DNA analysis and sequencing and for estrogen- and progesterone-receptor determinations. Corresponding slices from the other half were prepared for histopathologic examination. The part of the tumor that was frozen was stored at −70 °C until analysis.

Sequence-Based Analysis of p53 Status

RNA preparation and isolation. A 1.5-mL polypropylene microcentrifuge tube containing 300 μL of extraction solution (RNAzole, Cinna Biotec Inc, Houston, TX) was placed on wet ice. A section of frozen tumor specimen (5 × 2 × 2 mm) to be analyzed was removed with the aid of a disposable scalpel. During this procedure, care was taken to avoid thawing of the specimen to prevent RNA degradation. Such precaution was essential if repeated RNA preparation proved to be necessary. The excised tumor section was placed in the microcentrifuge tube, and it was pressed and squeezed against the walls of the tube with a disposable micropestle (Bergman and Beving, Stockholm, Sweden). Five hundred microliters of RNAzole and 80 μL of a mixture of chloroform and isoamyl alcohol (in the proportions of 24:1) were added to the tube. The contents of the tube were then mixed for 10 seconds using a vortex mixer, and the tube was returned to wet ice for 5 minutes to allow RNA to phase-separate from tissue and other cellular components. Subsequently, the tube was spun in a microcentrifuge for 10 minutes (at 14 000*g*), and 350 μL of the aqueous, upper phase was recovered and transferred to a new tube that contained 350 μL of isopropanol. After brief vortex mixing, the new tube was placed on wet ice for 30 minutes and then subjected to microcentrifugation at 14 000*g* for 20 minutes. The supernatant was discarded, and the pelleted RNA was washed twice with 70% ethanol, dried briefly, and finally dissolved in 50 μL of diethyl pyrocarbonate-treated water and 1 μL of RNA guard (25 U; Pharmacia Biotech AB, Uppsala, Sweden).

cDNA synthesis. The RNA samples were heat denatured at 90 °C for 3 minutes, followed by chilling on wet ice for 3 minutes. To prepare a cDNA reaction mixture, 25 μL of a given RNA sample was transferred to a microcentrifuge tube containing 10 μL of Moloney murine leukemia virus reverse transcriptase (200 U, Pharmacia Biotech AB), 2.5 μL of RNA guard (62.5 U), 37.5 μL of 2× "cDNA mix" (90 m*M* Tris–HCl [pH 8.3], 138 m*M* KCl, 18 m*M* $MgCl_2$, 30 m*M* DTT [dithiothreitol], 3.6 m*M* dCTP [deoxycytidine triphosphate], 3.6 m*M* dATP [deoxyadenosine triphosphate], 3.6 m*M* dTTP [deoxythymidine triphosphate], 3.6 m*M* dITP [deoxyinosine triphosphate], 0.9 m*M* dGTP [deoxyguanosine triphosphate], and 0.152 A_{260} U of pd$[N]_6$ random primers [approximately 2.5 pmol of primers]) to yield a final volume of 75 μL. The cDNA reaction mixture was incubated at 37 °C for 1 hour, and the reaction products were heat-denatured at 90 °C for 3 minutes and stored at −20 °C.

Polymerase chain reaction (PCR). Five microliters of 10× PCR II buffer (Perkin-Elmer AB, Sundbyberg, Sweden), 5 pmol each of the 5′ PCR primer and the 3′ PCR primer (one of them being biotinylated), 1.2 μL of 25 m*M* $MgCl_2$ (Perkin-Elmer AB), 28 μL of distilled H_2O, and 0.8 μL of Taq polymerase (4 U) (Ampli Taq, Perkin-Elmer AB) were mixed together in individual 0.2-mL PCR tubes (Perkin-Elmer AB). Five microliters of a given cDNA preparation or negative control template was added to specified tubes, yielding total PCR reaction volumes of 50 μL. The reaction mixtures were incubated in a Perkin Elmer 9600 PCR machine programmed to carry out 38 temperature cycles with the following profile: 94 °C, 15 seconds; 58 °C, 30 seconds; and 72 °C, 45 seconds. A 5-minute incubation at 72 °C was performed at the conclusion of the thermocycling program, followed by incubation at 4 °C.

Primers. PCR and DNA sequencing primers were synthesized based on the cDNA sequence of p53 messenger RNA. PCR primers were prepared by Custom Design Oligonucleotides (Pharmacia Biotech AB). Four sets of primers were used to cover the complete protein coding region of the p53 cDNA.

PCR primers (B = biotin-labeled). Fragment 1: B-5′-GAC ACG CTT CCC TGG ATT GGC-3′ and 5′-GCA AAA CAT CTT GTT GAG GGC A-3′ (covers the entire sequence of exons 2, 3, and 4 plus parts of exons 1 and 5). Fragment 2: B-5′-GTT TCC GTC TGG GCT TCT TGC A-3′ and 5′-GGT ACA GTC AGA GCC AAC CTC-3′ (covers the entire sequence of exons 5 and 6 plus parts of exons 4 and 7). Fragment 3: 5′-TGG CCC CTC CTC AGC ATC TTA-3′ and B-5′-CAA GGC CTC ATT CAG CTC TC-3′ (covers the entire sequence of exons 6, 7, 8, and 9 plus parts of exons 5 and 10). Fragment 4: 5′-CGG CGC ACA GAG GAA GAG AAT C-3′ and B-5′-CGC ACA CCT ATT GCA AGC AAG GG-3′ (covers the entire sequence of exons 9, 10, and 11 plus a part of exon 8).

DNA sequencing primers (fluorescein-labeled). Fragment 1:F-5′-CAG GGG AGT ACG TGC AAG TCA CAG-3′. Fragment 2:F-5′-GCC AAC CTC AGG CGG CTC ATA-3′. Fragment 3:F-5′-CGA GTG GAA GGA AAT TTG CGT-3′. Fragment 4:F-5′-GGG GAG CCT CAC CAC GAG CTG-3′.

Agarose gel electrophoresis and quality control. To control for contamination of PCR products that might have originated during the steps preceding DNA sequencing, we included negative control samples for both the RNA isolation and the cDNA preparation steps. The negative controls consisted of not adding tissue to RNA extraction tubes and not adding RNA to cDNA-reaction mixtures. The presence of amplified DNA in negative-control sample tubes was taken as an indication that all samples might be contaminated and that the corresponding batch of samples had to be discarded.

The purity, quality, and quantity of amplified DNA from specimens and from controls were evaluated by subjecting 5-μL aliquots of the relevant PCR products to electrophoresis in 1% agarose gels containing 5 μg/mL ethidium bromide. The 100 base-pair ladder (0.2 μg; Pharmacia Biotech AB) was used in the gels as a reference standard.

Solid-phase DNA sequencing on combs. Sequencing reactions were performed as described by Lagerkvist et al. (*24*) with the use of streptavidin-coupled Sepharose HP (AutoLoad, Pharmacia Biotech AB) attached to the teeth of plastic combs (solid-phase sequencing combs).

Forty microliters of a PCR product was transferred to a "4-teeth well" containing 80 μL of capture buffer (10 m*M* Tris–HCl [pH 7.5], 1 m*M* EDTA, and 2.0 *M* NaCl). The liquid was carefully mixed by pipetting (avoiding the generation of air bubbles), and a solid-phase sequencing comb was immersed in the resulting mixture. PCR products were captured by the comb during an incubation that lasted at least 60 minutes at room temperature. This incubation was interrupted only by occasional raising and dipping of the comb to improve PCR product capture (*24*).

The comb was then moved to a second "4-teeth well" containing 100 μL of 0.1 *M* NaOH and incubated at room temperature for 5 minutes. Subsequently, the comb was washed once with 0.1 *M* NaOH, once with TE buffer (10 m*M* Tris–HCl [pH 7.5] and 1 m*M* EDTA), and once with ultra-pure distilled H_2O. One hundred four microliters of distilled H_2O, 12 μL of 10× concentrated annealing buffer (AutoRead kit, Pharmacia Biotech AB), and 4 μL of a 1 pmol/mL stock solution of fluorescein-labeled sequencing primer were mixed in a third "4-teeth well," followed by insertion of the comb. This annealing mixture, with the comb immersed in it, was heated at 55 °C for 5 minutes and then placed at room temperature for at least 10 more minutes.

Twenty microliters of sequencing reaction mixture (containing 2 μL of 10× concentrated annealing buffer, 1 μL of extension buffer [AutoRead kit, Pharmacia Biotech AB], 4 μL of d/ddNTP mixture, 12 μL of distilled H_2O, and 1 μL of T7 DNA polymerase [2 U] [diluted in enzyme dilution buffer; AutoRead kit, Pharmacia Biotech AB]) were dispensed into individual "1-tooth wells" just prior to insertion of the comb. The comb was incubated in the wells for 5 minutes at 37 °C, and the entire well assembly (with the comb still inserted) was placed finally on wet ice.

The sample wells of an automated laser fluorescence (ALF; Pharmacia Biotech AB)-sequencing gel (containing 6% polyacrylamide and 7 *M* urea) were rinsed with 1 × TBE buffer (100 m*M* Tris, 90 m*M* boric acid, and 1 m*M* EDTA [pH 8.3]), prewarmed to 45 °C, and loaded with 15 μL of 100% formamide STOP solution (AutoRead kit, Pharmacia Biotech AB). The comb was removed from the sequencing-reaction wells and inserted into the wells of the ALF-sequencing gel for 10 minutes. The comb was then carefully removed from the gel apparatus, and ALF electrophoresis was initiated.

Sequencing evaluation and verification. Evaluation of p53 sequences was performed with the aid of a newly developed software program, Sequence Evaluator (Pharmacia Biotech AB), version 0.16, which compares the wild-type sequence of a gene with sequences obtained from sample analysis.

All mutations were confirmed by reamplifying the relevant cDNAs and sequencing the new PCR products. If a mutation was identified in a region contained within the overlapping portion of two cDNA segments, its existence was further verified by analyzing amplification products from the neighboring cDNA segment.

Immunohistochemical Analysis of p53 Status

Paraffin-embedded tumor sections on silane-coated slides (Eire Scientific Co., Porthmouth, Philadelphia, PA) were dewaxed in xylene and rehydrated in ethanol and distilled water. Pretreatment in a microwave oven at 750 W (three times for 5 minutes each) enhanced p53 antigen accessibility to antibodies. The mouse monoclonal antibody cl 1801 (*12*) (Biozac AB, Järfälla, Sweden), which recognizes both wild-type and mutant forms of p53, was used at a dilution of 1:100.

Immunostaining was performed in a Ventana ES Automated Immunohistochemistry Instrument (Annex, Helsinki, Finland). We used the manufacturer's diaminobenzidine (DAB) detection kit that includes biotin-labeled secondary antibodies directed against mouse immunoglobulins, avidin-labeled horseradish peroxidase, and DAB as the localization reagents. Negative control IHC reactions were performed by omitting the primary antibody.

Positive p53 IHC staining is seen in the nucleus of cancer cells. In our primary IHC analysis, positive p53 staining in any percentage of cancer cells was considered to be positive.

In a secondary IHC analysis, IHC-positive samples were subclassified with regard to immunostaining intensity and extent according to graded scales that ranged from 1 to 3. For intensity of staining, 1 represented weakly positive tumor cells and 3 represented strongly positive cells. For extent of staining, 1 denoted samples in which less than one third of the tumor cells had positive staining, 2 signified those with positive staining in one third to two thirds of the tumor cells, and 3 denoted those with more than two-thirds positive staining. The results obtained with the two scales were multiplied against each other, yielding a single scale with steps of 1, 2, 3, 4, 6, and 9, where 1 and 2 were considered to be low staining, 3 and 4 were considered to be medium staining, and 6 and 9 were considered to be high staining. This classification system is derived from that described by Busch et al. (*25*). All slides were viewed and judged independently by two pathologists (A. Lindgren and H. Nordgren) according to this multiplied scale at different times without knowledge of clinical outcome and p53 mutation status.

Statistical Methods

Survival probabilities for overall, breast cancer-corrected, and relapse-free survivals were estimated by use of the Kaplan–Meier method, and the equality of survival curves for different subgroups was evaluated by use of the logrank method. All *P* values are estimated from two-sided statistical tests. Relative hazards of dying of breast cancer were estimated by use of Cox's proportional hazards models. In the multivariate models, age at diagnosis, tumor size, estrogen- and progesterone-receptor status, and S phase proportion were taken into consideration. Hormone-receptor status was dichotomized as negative versus positive variables (cutoff point, 0.1 fmol/μg DNA) and S phase as high versus low variables (cutoff points, 7% for diploid and 12% for aneuploid tumors, respectively). In breast cancer-corrected survival, death from breast cancer was considered to be the event of interest; all other deaths were treated as censoring

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points. Thus, in the breast cancer-corrected survival analysis, all patients with causes of death other than breast cancer were excluded.

Results

Clinical Outcome

Of the 316 patients included in the study, 48 died of breast cancer, five died with breast cancer present, and 21 died of unrelated causes. For seven patients, we lack information about the cause of death. The median follow-up in this study was 57 months, with a maximum follow-up of 87 months.

Mutations Detected and IHC Results

Alterations in the p53 gene were detected by means of the cDNA-based sequencing method in tumors from 69 (22%) of the 316 patients. p53 mutations were found throughout the entire protein coding region of the gene. Twenty-nine p53 mutations were detected in patients whose tumors had metastasized to axillary lymph nodes, 37 mutations were found in node-negative patients, and three mutations were detected in patients with unknown primary lymph node status. We identified 45 missense (simple point) mutations, seven nonsense mutations (creating premature stop codons), five in-frame deletions, eight out-of-frame deletions, one in-frame insertion, and three out-of-frame insertions (Table 1; Fig. 1). Thirty-one (45%) of the 69 mutations were located in evolutionarily conserved regions of p53 (Fig. 1). From four tumors (1.3% of the total), we were unable to obtain sequence information; from three others (1% of the total), we lack immunohistochemical data (Table 2). Thus, tumors from 309 patients were available for this comparative study. Since follow-up data were missing for one patient, 308 patients could be included in survival analyses.

Positive IHC (suggesting p53 mutation) was demonstrated in tumors from 64 (20%) of the 316 patients. Twenty-three (33%) of the sequence-positive patients (i.e., proven to have p53 mutations) were negative by IHC, whereas 19 (30%) of the IHC-positive patients were sequence negative (Table 2). Negative immunohistochemical reactions were noticed in all six tumors with mutations that created premature stop codons and in 11 (85%) of 13 tumors with deletions (Table 1; Fig. 1). Positive immunohistochemical reactions were seen in 40 (89%) of 45 tumors with point mutations (Table 1; Fig. 1).

The primary method of IHC classification (*see* "Materials and Methods" section) was used to generate the IHC data described above. There was complete concordance between the two pathologists regarding the assessment of negative and positive immunohistochemical reactions with this method.

When the 64 immunohistochemically positive tumors were subclassified according to the 6-graded (1-9) scale (i.e., our secondary IHC analytical method; *see* "Materials and Methods" section), the pathologists agreed in 75% of the cases. With this more complex grading system, 12.5% of the tumors fell into different low-, medium-, and high-staining groups as judged by the two independent investigators, whereas assessments regarding low-staining versus medium- to high-staining were divergent in only 7.8% of cases. Within the low-staining group, however, there was a major discrepancy regarding subclasses 1 and 2, and there was only 53.8% agreement with respect to the proposed class-1 tumors.

Comparison of Survival Data

We analyzed the patient data with regard to survival and p53 mutation status as determined by IHC and by cDNA-based sequencing. Survival was illustrated as relapse-free survival (RFS; 304 patients), breast cancer-corrected survival (BCCS; 308 patients), and overall survival (OS; 308 patients).

IHC and survival. According to IHC, there was a trend of reduced OS for patients with p53-positive tumors compared with those having p53-negative tumors, but it did not reach statistical significance ($P = .2$) (Table 3). No statistically significant differences could be detected in any of the survival parameters between p53-positive and p53-negative tumors according to IHC (Table 3).

cDNA sequencing and survival. Highly significant differences in survival were seen between patients with sequencing-positive and sequencing-negative tumors, with worse prognosis for those with positive tumors. The 5-year OS frequency was 78% in the mutation-negative group as opposed to 55% for those with p53 mutations ($P = .0003$). Similar statistically significant differences were seen for RFS and BCCS (Table 3).

Positive IHC with or without positive cDNA sequencing. In 61 women with positive IHC, we saw statistically significant differences in RFS between those with sequencing-positive tumors and those with sequencing-negative tumors (Fig. 2, top panel). The 5-year RFS in this IHC-positive patient population was 86% for the sequencing-negative group and 56% for the sequencing-positive group ($P = .02$; Fig. 2, top panel). Differences in BCCS between sequencing-positive and sequencing-negative patients with positive IHC were also statistically significant (62 patients evaluated, data not shown). None of 19 patients with IHC-positive tumors but negative-sequencing results had died of breast cancer (BCCS) at 60 months' follow-up, whereas 10 of the 43 patients with positive-sequencing results had died at 60 months of follow-up ($P = .03$). There was a trend in the same direction for OS, but it was not statistically significant (Fig. 3, top panel).

Negative IHC with or without positive cDNA sequencing. In the 246 evaluable patients with negative IHC, there were statistically significant differences in all three survival categories, including RFS (243 patients; Fig. 2, bottom panel), between the sequencing-negative and sequencing-positive cases, with worse prognosis for patients with positive sequencing results. The most marked difference was seen in the OS comparison, where the 5-year survival frequency was 78% in the sequencing-negative group as opposed to 48% in the sequencing-positive group ($P = .002$; Fig. 3, bottom panel).

Positive cDNA sequencing with or without positive IHC. For the women with sequence-determined p53 mutations, there were no significant differences in any of the survival categories between the IHC-positive and the IHC-negative groups (Fig. 2, left panel; Fig. 3, left panel).

Negative cDNA sequencing with or without positive IHC. For the cDNA-based sequencing-negative patients, no significant differences in survival were observed between the IHC-positive and the IHC-negative groups. There was even a trend

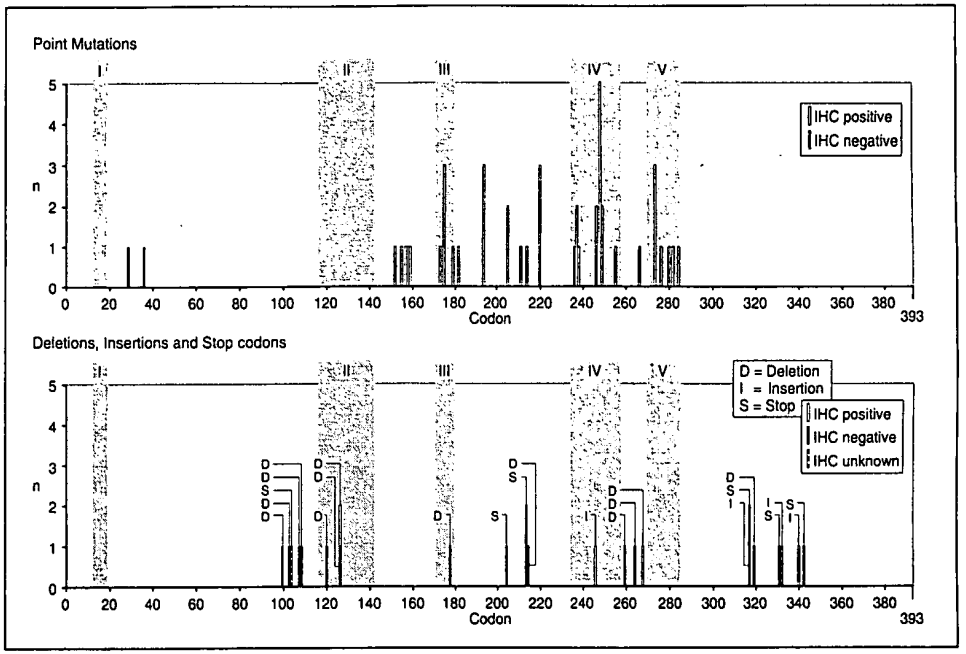

Fig. 1. Detection of mutant p53 by immunohistochemistry (IHC). Ability to detect p53 coding region point (missense) mutations (upper panel), deletions, insertions, and premature stop codons (lower panel) with the monoclonal antibody Pab 1801. Codon positions are outlined below the *X* axis. The numbers (n) of individual p53 alterations are given on the *Y* axis. The gray-shaded areas indicate evolutionarily conserved regions I-V [*see* (22) and references contained therein]. p53 alterations identified by complementary DNA sequencing but not by IHC are indicated by filled black bars, whereas those identified by both methods are indicated by unfilled bars; alterations with unknown IHC status are indicated by bars with alternating black and white filling.

Table 1. Different types of p53 mutations detected by complementary DNA sequencing analysis and corresponding anti-p53 IHC analysis*

	Point mutations	Deletions		Insertions		Premature stop codons	Total
		In frame	Out of frame	In frame	Out of frame		
IHC +	40	2	0	1	1	0	44
IHC −	5	3	8	0	1	6	23
Unknown IHC	0	0	0	0	1	1	2
Total	45	5	8	1	3	7	69

*IHC = immunohistochemistry.

Table 2. No. of tumors positive and negative for p53 mutation according to IHC- and complementary DNA sequence-based determinations*

		Sequence-based determination of p53 status			
		Mut	Wt	Unknown	Total
IHC p53 detection	Positive	44	19	1	64
	Negative	23	223	3	249
	Unknown	2	1	0	3
	Total	69	243	4	316

*IHC = immunohistochemistry; Mut = mutation-positive tumors; Wt = wild-type p53.

Table 3. 5-year survival in relation to p53 mutation status detected by cDNA sequencing and immunohistochemistry (IHC), respectively*

	5-y survival					
	cDNA sequencing			IHC		
p53 status	RFS, % (n)	BCCS, % (n)	OS, % (n)	RFS, % (n)	BCCS, % (n)	OS, % (n)
Positive	54 (66)	69 (66)	55 (66)	65 (61)	81 (62)	65 (62)
Negative	71 (238)	86 (242)	78 (242)	68 (243)	83 (246)	75 (246)
P	.001	.01	.0003	.5	.8	.2

*RFS = relapse-free survival; BCCS = breast cancer-corrected survival; OS = overall survival; n = No. of patients; positive = mutation positive by sequencing or positive staining by IHC; negative = wild-type p53 or negative staining by IHC; cDNA = complementary DNA. All *P* values according to the logrank test.

for *better* survival in the group with positive IHC reactions (Fig. 2, right panel; Fig. 3, right panel).

Survival Analysis When IHC Low-Staining Tumors Were Classified as p53 Negative

In our primary IHC analysis of p53 status and survival, which did not indicate any statistically significant differences in survival between IHC-positive and IHC-negative groups, all 64 tumors with positive IHC staining were considered to be p53 positive (i.e., mutant). We then reanalyzed the data obtained from each of the pathologists, considering the IHC-positive subclasses 1-2 (i.e., the "low-staining group" in the more complex grading system, *see* "Materials and Methods" section) as negative cases. In comparison with our primary classification, this secondary classification improved all *P* values for differences in RFS, BCCS, and OS between IHC-positive and IHC-negative

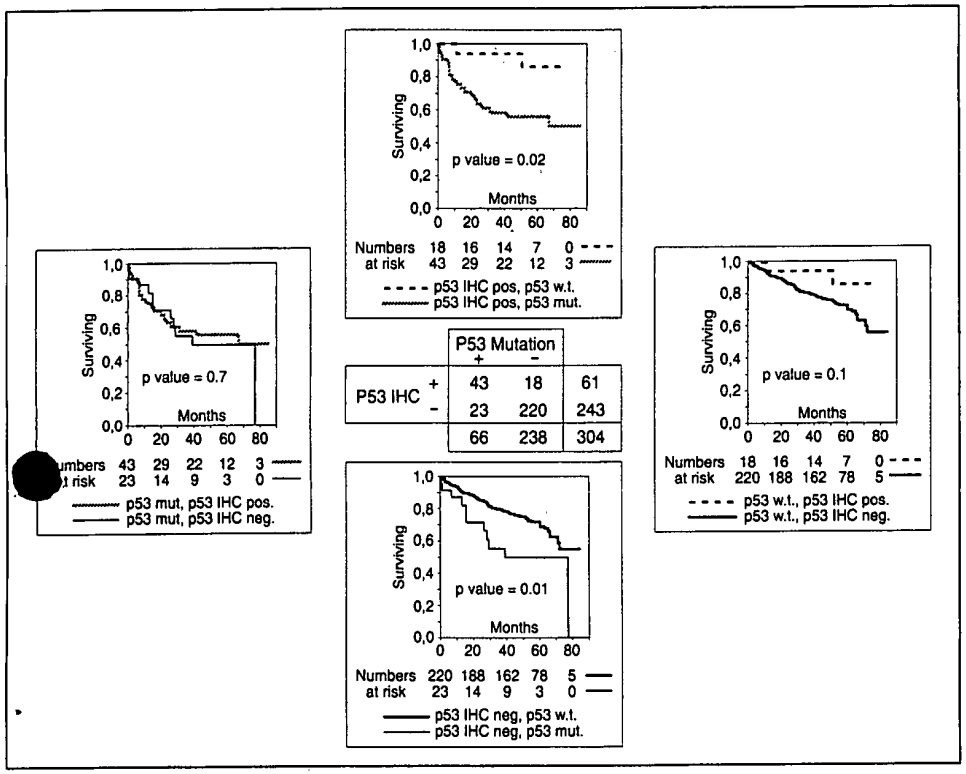

Fig. 2. Relapse-free survival for patients with breast cancer categorized according to p53 complementary DNA sequencing and immunohistochemistry (IHC) data. The four diagrams (top, left, bottom, or right) are based on a 2 × 2 design. Survival curves were generated according to the Kaplan–Meier method; statistical comparisons were made by use of the logrank method. w.t. = wild-type; mut = mutant; neg = negative; pos = positive.

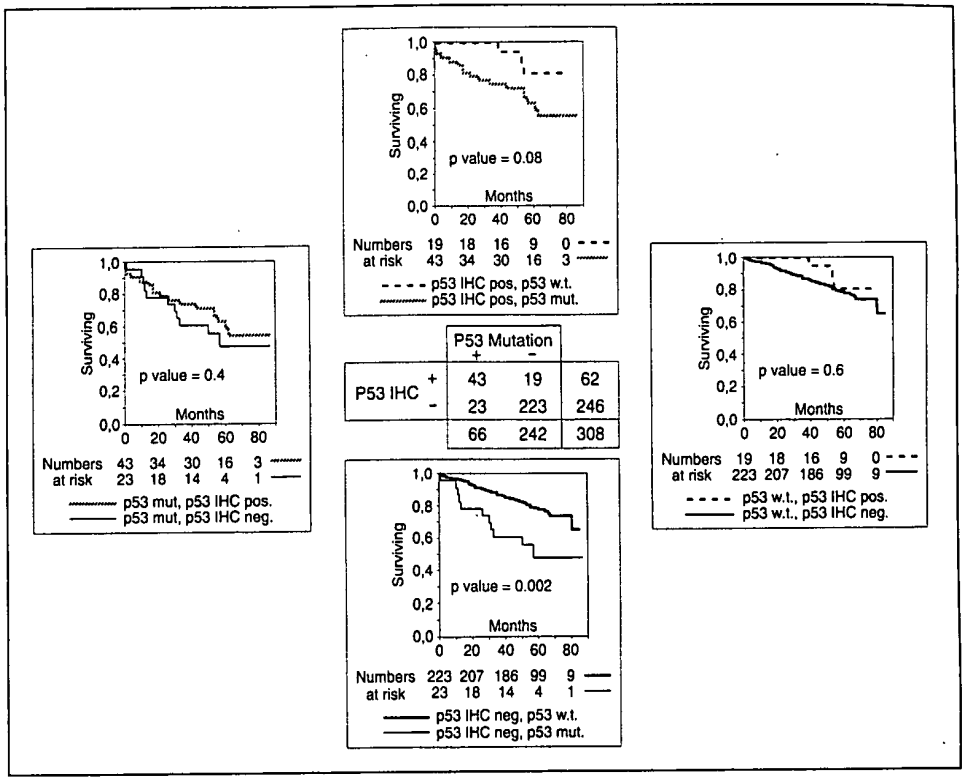

Fig. 3. Overall survival for patients with breast cancer categorized according to p53 complementary DNA sequencing and immunohistochemistry (IHC) data. The four diagrams (top, left, bottom, or right) are based on a 2 × 2 design. Survival curves were generated according to the Kaplan–Meier method; statistical comparisons were made by use of the logrank method. w.t. = wild-type; mut = mutant; neg = negative; pos = positive.

tumors. The best *P* values were seen for OS and, according to data from one of the pathologists, the difference in OS became statistically significant (Table 4). The *P* values obtained from the other pathologist's review were also improved relative to those from the primary analysis, but none of the values reached statistical significance. However, it is important to note that with this modification in IHC classification the number of positive cases was reduced to 36 and 39, as judged by the two pathologists, respectively (Table 4).

Proportional Hazards Models

Proportional hazards models were tested to investigate whether prognostic information generated by cDNA sequencing and IHC was positively or negatively confounded by other commonly used prognostic markers. The relative hazards for p53 alterations were of the same magnitude in both the univariate and multivariate models (Table 5). The relative hazards for positive cDNA sequencing data were higher than those for positive IHC data, with confidence intervals indicating an independent effect; the confidence intervals for the immunohistochemical data clearly included 1.0.

Table 4. Overall survival at 5 years in relation to p53 status determined by cDNA sequencing, IHC (primary analysis*), and IHC modified by subclassification (secondary analysis*)†

	5-y overall survival		
	p53 status		
	Positive	Negative	
Method	% (n)	% (n)	*P*
Sequencing	55 (66)	78 (242)	.0003
IHC	65 (62)	75 (246)	.2
IHC 1	52 (36)	76 (272)	.01
IHC 2	59 (39)	75 (269)	.05

**See* "Materials and Methods" section for details.

†IHC = immunohistochemistry; cDNA = complementary DNA; sequencing = cDNA sequencing; p53 status positive = mutation by sequencing or positive IHC; p53 status negative = wild-type p53 by sequencing or negative IHC; IHC 1 = IHC results by pathologist 1 (A. Lindgren) after modification with subclasses 1 and 2 considered to be negative IHC; IHC 2 = IHC results by pathologist 2 (H. Nordgren) after modification with subclasses 1 and 2 considered to be negative IHC. All *P* values according to the logrank test.

Table 5. Results from Cox's proportional hazards models*

Factor	Univariate	Multivariate 1	Multivariate 2
p53 mut versus wt	2.1 (1.1-3.8)	1.9 (1.0-3.7)	—
p53 IHC + versus IHC –	1.2 (0.9-1.5)	—	1.2 (0.9-1.6)
Tumor size	—	1.0 (0.99-1.02)	1.0 (0.99-1.02)
Node + versus node –	—	5.0 (2.6-9.9)	5.2 (2.6-10.1)
ER + versus ER –	—	1.0 (1.0-1.04)	1.0 (1.0-1.04)
PR + versus PR –	—	1.0 (0.93-1.01)	1.0 (0.94-1.01)
S phase, high versus low	—	1.5 (0.7-2.9)	1.5 (0.7-3.0)

*Mut = mutation; wt = wild-type; IHC = immunohistochemistry; + = positive; – = negative; node = axillary lymph nodes; ER = estrogen receptor; PR = progesterone receptor. Estimates of relative hazards with 95% confidence intervals for breast cancer-corrected survival. Multivariate model 1 estimates the effect of p53 mutation determined by complementary DNA sequencing; model 2 estimates the relative hazard for IHC data.

Discussion

This study differs from most other studies of p53 status and cancer in that the patient population, consisting of 316 women with primary breast cancer, was derived from a population-based cohort. The breast cancer specimens were examined by IHC with use of the monoclonal antibody Pab 1801, an antibody whose use on paraffin-embedded tumor samples is now widely accepted (*7,16,26-29*). The specimens were also analyzed by use of a cDNA-sequencing strategy in which all exons of the p53 gene were evaluated. Our cDNA sequencing approach stands in contrast with previous investigations of p53 gene alterations in human breast cancer, which have focused primarily on sequence changes in exons 5, 6, 7, and 8.

We observed that 23 breast cancers with p53 alterations detected by cDNA-based sequencing failed to generate positive IHC reactions with Pab 1801; 19 tumors were negative for mutation by cDNA sequencing but were positive by IHC. The lack of concordance between results obtained with these two methods may indicate that they measure different aspects of p53. An ideal method should be one that gives the best prognostic information in relation to currently used therapeutic approaches and the best delineation of the patient groups studied. Our data clearly demonstrate that the cDNA sequencing method is superior in these regards. Our finding should be of importance since most published p53 status determinations have been based on IHC analyses. In view of the discrepancy between results obtained with the two methods, we will now discuss possible explanations for false-positive and false-negative results with each method.

The prognostic information generated by IHC- and cDNA sequence-based determinations of p53 status gave us reason to believe that IHC might generate false-positive as well as false-negative results. This was suggested by the observation that the patient group with negative cDNA sequencing data (i.e., their tumors had wild-type p53 genes) but positive IHC results did not seem to have a significantly worse prognosis than the corresponding IHC-negative group. The suspicion of false-positive IHC results is supported when considering the IHC-positive group, where comparison of the sequencing-positive and sequencing-negative patients showed significantly better survival for the patients with negative sequencing data. There are also signs of false-negative cases of IHC in the IHC-negative patient group, where significantly worse prognosis was observed for patients with positive cDNA sequencing data compared with those with negative sequencing data. Similarly, in the cDNA sequencing-positive group, no difference in prognosis was seen between the IHC-positive and the IHC-negative patients, supporting our conclusions.

False-negative IHC results may be generated as a consequence of premature stop codons and gross deletions in the p53 gene, since such alterations could lead to a cessation of protein synthesis and render the detection of mutations by IHC impossible. Our results are consistent with this hypothesis, since all six samples with premature stop codons showed negative IHC reactions. Similarly, 11 of 13 tumors with deletions and one of three tumors with insertions were negative by IHC. Thus, 18 of the 23 evaluable samples with aberrations detected by cDNA sequencing but IHC negative had deletions, insertions, and premature stop codons. In contrast, only four of 44 samples with positive IHC had mutations of these types ($P<.001$).

Another possible explanation for negative IHC results in sequencing-positive tumors may be that the genetic alterations caused changes in or disappearance of the epitope recognized by Pab 1801, which is located between amino acids 40 and 65 (*30*). However, few of the alterations that we identified are located in this region. Furthermore, most of the p53 proteins truncated as a consequence of premature stop codons should still contain the Pab 1801 epitope. It has been suggested that missense mutations (*19,31*), as well as deletions, insertions (*32*), and premature stop codons (*33*), might produce conformational changes in the p53 polypeptide that interfere with recognition of the epitope for Pab 1801. In addition, Ohuc et al. (*33*) have proposed that truncation of the carboxyl terminus of p53 might reduce the stability of the mutated protein because of the loss of several important functional domains, such as the DNA binding domain, the nuclear localization signals, and the oligomerization domain. The accumulation of p53 protein would thus fail to occur, making detection by IHC impossible. In this study, all deletions and stop codon mutations identified were located downstream of codons that define the Pab 1801 epitope, supporting these theories. It is also possible that certain point mutations may not be able to stabilize the p53 protein sufficiently to be detectable by IHC (*21*).

The theoretical basis for the determination of p53 status by IHC is that mutant p53 protein exhibits a longer half-life than wild-type p53, which results in the accumulation of p53 protein in transformed cells (*4,19*). However, it is possible that the accumulation of p53 in tumor cells may, in some cases, indicate the existence of a regulatory defect rather than mutations in the protein-coding sequence of the gene. Several investigators (*34,35*) have found discrepancies between p53 protein expression and mutation status. In some cases, weak immunostaining could also represent normal cell cycle fluctuations in p53 protein levels, as indicated in a few reports (*36,37*). In our study, 13 of 19 tumors that were positive for p53 by IHC and had wild-type p53 cDNA sequences displayed weakly positive IHC signals only. For the 243 patients with negative cDNA sequencing data, we observed a nonsignificant trend of better survival for those with IHC-positive tumors relative to those with IHC-negative tumors. One may speculate that the improved survival could be due, in part, to increased amounts of normal p53 protein, which might facilitate apoptosis induced by tamoxifen therapy, chemotherapy, or radiotherapy.

Can IHC analysis be refined to increase its resolving power? It has been shown that strong immunostaining is associated with the presence of p53 gene alterations detected by molecular biological methods to a greater extent than is low-grade IHC staining (*38*). It has also been demonstrated that IHC with Pab 1801 generates better prognostic information when considered as a continuous variable than as a dichotomous variable (*8*).

With these thoughts in mind, we made an additional analysis in which the IHC low-staining group (i.e., subclasses 1 and 2) were considered to be negative. This approach improved the significance of the P values obtained in all three measured survival categories. One of the P values, on the basis of one pathologist's data, became statistically significant (OS, IHC positive versus IHC negative), whereas in our primary analysis (where all positive reactions were considered as p53-positive cases) none of the P values were significant. This result may indicate that the diagnostic specificity of IHC is impaired by technical limitations and/or more fundamental biological properties of the p53 regulatory system. To achieve a similar level of significance as that obtained with the use of sequencing data, the number of positive cases was substantially reduced, thus lowering the sensitivity of the method and increasing the risk of false-negative cases.

Having analyzed the IHC data with regard to false-negative and false-positive results, we will now discuss the sequence-based analysis in the same respect. False-positive sequencing results may occur as a consequence of contamination of samples during processing. In this study, we identified p53 gene mutations in 69 cases. Most of these alterations were found in 49 different codons resulting in 55 different mutations. Seven of the 14 remaining mutations were located in mutational "hot spots" reported by others (*39*). Given this diversity, we have no reason to suspect false-positive sequencing data in our study. This conclusion is further strengthened by the finding that none of the negative cDNA/PCR controls yielded any amplification products, indicating that the integrity of the tumor isolates was maintained.

False-negative sequencing reactions may, on the other hand, represent a greater risk. Theoretically, this could happen if the tumor samples used for the analysis contained relatively few malignant cells in relation to normal cells, which could cause the wild-type sequence to "drown out" the mutant sequence. The manner in which the tumor material was isolated in this study should have minimized such a risk. cDNA sequencing might also fail to detect a mutation if the alteration is located in a position disadvantageous for proper primer (cDNA or PCR) binding. This risk would be greatest at the extreme ends of the p53 coding region with our approach.

Taken together, our data indicate that direct cDNA sequence-based analysis of p53 status is superior to IHC in determining prognosis in breast cancer. If complete sequencing of the p53 coding region by use of the present method is taken as the gold standard, false-positive as well as false-negative results can occur with IHC. In terms of using p53 status in clinical decision making with regard to adjuvant therapy, both false positives and false negatives would pose problems.

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Differences in Lung Cancer Risk Between Men and Women: Examination of the Evidence

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Background: **Lung cancer incidence is gradually leveling off in U.S. men but is continuing to rise in U.S. women. This increase in U.S. women exceeds that expected from a slower decline of smoking among women. Recent epidemiologic and biochemical studies suggest gender differences in susceptibility to tobacco carcinogens.** ***Purpose:*** **We conducted an up-to-date, more in-depth evaluation of our earlier observation of a potential gender difference in relative risk (RR) of lung cancer due to smoking. We added information from several additional case and control subjects and included more precise histologic classification of the cancer type, accurate quantitation of smoke exposure, and adjustments for body size.** ***Methods:*** **The present investigation was a part of an ongoing hospital-based, case–control study by the American Health Foundation. It included data from 1889 case subjects (1108 males and 781 females) with lung cancer of squamous/epidermoid, small-cell/oat cell, large-cell, and adenocarcinoma types and 2070 control subjects (1122 males and 948 females) with diseases unrelated to smoking. The case and control subjects were admitted to participating hospitals from 1981 to 1994 and were pair-matched by age, sex, hospital, and the time of hospital admission. Ex-smokers and non-Caucasians were excluded from analyses to avoid confounding. The RRs and 95% confidence intervals were estimated from adjusted odds ratios (ORs) by use of unconditional multiple logistic regression analysis, and statistical significance was determined by two-sided tests. The ORs for major histologic types were estimated at increasing levels of exposure to cigarette smoke.** ***Results:*** **Our results indicated that women were more likely to be never-smokers than men, particularly those with the squamous/epidermoid-type cancer (8.3% for women versus 2.9% for men 55 years old or older). Men started smoking earlier, reported inhaling more deeply, and smoked more cigarettes per day than women. In contrast, dose–response ORs over cumulative exposure to cigarette smoking were 1.2-fold to 1.7-fold higher in women than in men for the three major histologic types; these differences were more pronounced for small-cell/oat cell carcinomas and adenocarcinomas than for squamous/epidermoid carcinomas. Adjustments for weight, height, or body mass index did not alter the ORs.** ***Conclusions:*** **These results confirm our earlier finding that the ORs for major lung cancer types are consistently higher for women than for men at every level of exposure to cigarette smoke. Furthermore, this gender difference cannot be explained by differences in base-line exposure, smoking history, or body size, but it is likely due to the higher susceptibility to tobacco carcinogens in women. [J Natl Cancer Inst 1996;88:183-92]**

It is a well-established fact that cigarette smoking is the principal cause of lung cancer in both men and women. The continued higher incidence rates in men reflect their longer and greater exposure to cigarette tar (*1*).

A pattern has evolved during the past decade in the United States showing that, while lung cancer incidence is leveling off among men, it is continuing to rise at a steady rate among women (*2*). In fact, there has been a 500% increase in female lung cancer mortality since 1950 (*3*), surpassing breast cancer as the leading cause of cancer deaths among U.S. women since 1987 (*4*). At the same time, because of the slower decline in smoking prevalence among women than among men (*1*), the exposure of women to tobacco carcinogens has gradually approached and, in fact, may soon surpass that of men (*2*). Consequently, if current trends continue, the lung cancer rates among women are expected to surpass those among men within the next two to three decades.

In light of these trends, recent epidemiologic findings (*5-13*), which suggest that, dose for dose, women may be more susceptible to tobacco carcinogens than men, are of concern. In fact, the rate of decrease in the gap between male–female lung cancer rates observed during the past three decades is more pronounced than would be expected on the basis of the changing trends in male and female smoking rates alone. Although the issue of a higher susceptibility to tobacco carcinogens by female smokers is still inconclusive, the potential public health consequences of such a phenomenon would be substantial.

Our previous work (*8*), suggesting that women may be more susceptible to tobacco carcinogens than men, was limited to a broad histologic classification of lung cancer, i.e., Kreyberg I and Kreyberg II types. It is important to further evaluate this finding by using more precise histologic subtypes. In 1985, we started collecting more detailed smoking histories from the study participants. As a result, we now have more precise quantitation of lifelong smoking exposure for each participant based on as many as seven different brands of cigarettes smoked.

Spurred by our initial findings (*8*) and by the availability of additional data on more case subjects as well as control subjects (with more detailed and precisely quantitated smoking exposures and more defined lung cancer histologies), we conducted an in-depth evaluation of the differences in lung cancer risk between men and women. By reviewing the results of

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